REMARKS

Claim Amendments

Claims 1, 3-21, 29-32 and 34-52 are pending in the application. Claims 18-21, 51 and 52 stand withdrawn. Claims 1, 3-14, 16, 17, 29-32 and 34-50 stand rejected, and claims 2, 15 and 33 were indicated to be allowable if written in independent form.

Claims 1, 3, 8, 13, 16 and 32 are amended herewith. The limitations of claims 2 and 33 have been incorporated into claims 1 and 32, respectively. Claim 1 has also been amended to be consistent with the Examiner's restriction requirement as discussed in the Office Action. In particular, the Office Action indicates that the elected invention is directed to methods of reducing the risk of bacterial infection in individuals already colonized with bacteria. Applicants respectfully submit that previously filed claim 1 was not limited to such individuals. However, Applicants have amended claim 1 to recite a method for reducing the risk of bacterial infection or sepsis in a person colonized with pathogenic bacteria. Support for the amendment to claim 1 may be found, for example, in the combination of page 11, lines 6-17 and page 15, lines 15-32, as well as in original claims 1, 2 and 4. Claims 2, 4 and 33 have been canceled as duplicative. Applicants reserve their right to prosecute in one or more divisional applications methods directed to persons that are neither colonized or infected with a pathogenic bacteria as disclosed and claimed in original claim 1.

Support for amended claims 3, 8, 13, 16 and 32 can be found throughout the specification and in the claims as originally filed. *See, e.g.*, page 15, lines 1-32; page 19, lines 15-19; original claim 33. Applicants respectfully request entry of the above amendment and submit that the above amendment does not constitute new matter.

Restriction

Claims 51 and 52 have been withdrawn as allegedly being drawn to a non-elected invention. The Office Action states:

> The elected invention is drawn to a method of reducing the risk of bacterial infection, wherein bacteria have already colonized the patent, while claims 51 and 52 are drawn to a method of reducing the risk that persons who have not been colonized will acquire pathogenic bacteria. ***The patients are at different stages.*** Office Action, page 3. (emphasis added).

Applicants respectfully disagree and traverse this restriction. Applicants submit that claim 51 requires the administration of bacteriophage to colonized patients. Indeed, claim 51 recites, "treating a ***patient*** with bacteriophage preparation containing bacteriophage of one or more strains which produce lytic infections in pathogenic bacteria, ***wherein said patient is colonized*** with the pathogenic bacteria subject to infection by said bacteriophage." (emphasis added). Applicants acknowledge that claim 51 is directed to a method of reducing the risk that pathogenic bacteria will be acquired by persons being neither colonized or infected with a pathogenic bacteria. The step of providing bacteriophage to a colonized patient, "wherein bacteria have already colonized the patient" is required in both the amended claims and claim 51. Applicants note that claim 51 could be rewritten as dependent on claim 32. Accordingly, Applicants respectfully request that claims 51 and 52 be rejoined with the elected claims.

Claim Rejections — 35 U.S.C. § 112, ¶ 2

Claim 16 stands rejected as being indefinite over the recitation of "the selected bacteria." Applicants have amended claim 16 to recite "the selected pathogenic bacteria" as suggested by the Examiner.

In view of the foregoing, Applicants respectfully request withdrawal of the rejection under 35 U.S.C. § 112, ¶ 2.

Claim Rejections — 35 U.S.C. § 102(b)

Claims 1, 4, 5, 7-9, 32, 35, 37-39 and 48-50 stand rejected as allegedly being anticipated by U.S. Patent No. 6,056,954 ("the '954 patent").

The Office Action asserts that the '954 patent teaches a method of using lytic bacteriophages[1] for preventing those who have been exposed to others who are sick, from becoming infected. Office Action, page 4.

Applicants respectfully disagree and traverse this rejection.

The claims relate to various methods comprising providing a patient with a composition containing bacteriophage of one or more strains which produce lytic infections in pathogenic bacteria.

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[1] Lytic bacteriophage, as described in the present specification, denote bacteriophage which lyse host bacteria without integrating into the host genome in contrast to lysogenic bacteriophage which may integrate into the host genome.

The '954 patent does not teach such methods. Rather, the '954 patent is directed to a method of treating bacterial diseases with lytic ***enzymes*** produced in bacteria infected with bacteriophage.[2] Bacteriophage do not contain lytic enzymes.[3] Indeed, lytic enzymes are produced in infected bacteria late in the infection cycle. The '954 patent formulates and uses the enzyme for treatment, not the bacteriophage itself. Accordingly, because the '954 patent does not teach a method comprising treating a patient with a composition comprising bacteriophage, the '954 patent does not anticipate the claimed invention.

Nonetheless, without acquiescing to the correctness of the rejection, Applicants have amended claims 1 and 32 to incorporate the limitations of claims 2 and 33, respectively. Applicants reserve their right to pursue the subject matter of previous claims 1 and 32 in a continuation application.

Claim Rejections — 35 U.S.C. § 103(a)

Claims 3, 6, 10-14, 17, 29-31, 34, 36 and 40-47 stand rejected as allegedly being unpatentable over the '954 patent, as applied to claims 1, 4, 5, 7-9, 32, 35, 37-39 and 48-50 above, and in view of Carlton et al. (Archivum Immunologiae et Therapiae Experimentalis, 47: 267-274, 1999, hereinafter "Carlton") and Risi et al. (Am J. Infect. Control, 26: 594-604, 1998, hereinafter "Risi").

The Office Action reiterates the asserted teachings of the '954 patent, but acknowledges that the '954 patent does not teach that "the patient is immunocompromised, the pathogenic bacteria are VRE or MDRSA or multi-drug resistant *Pseudomonas*, the composition can contain a plurality of bacteriophage strains to produce lytic infections against a plurality of bacterial species, or that the method can also be used to reduce the incidence of bacterial infections of patients admitted to a hospital." Office Action, pages 5

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[2] *See, e.g.*, col. 1, lines 12-16 ("The present invention discloses a method and composition for the ***treatment of bacterial infections by the use of a lysing enzyme blended with an appropriate carrier...***"); col. 2, lines 38-41 ("Consequently, the present invention discloses the extraction and ***use of a variety of bacterial phage associated lytic enzymes for the treatment*** of a wide variety of illnesses caused by bacterial infections."); col. 3, line 64 to col. 4, line 1 ("The method for treating bacterial infections comprises treating the infection with a therapeutic agent ***comprising an effective amount of at least one lytic enzyme produced by a bacteria infected with a bacteriophage specific for the bacteria.***).

[3] *See, e.g.*, Mathews, C. K. (1994). An overview of the T4 developmental program. Molecular Biology of Bacteriophage T4. K. J. D. Washington, D.C., American Society for Microbiology: 1-8, attached hereto as **Exhibit A**. Lytic proteins produced in bacteria at the end of the infection cycle are discussed on page 8, col. 2 of Mathews.

and 6. The Office Action cites Carlton for the proposition that phage therapy is used to treat VRE, but concedes that Carlton does not teach phage therapy for immunocompromised patients, or treating MDRSA or multi-drug resistant *Pseudomonas*. *Id.* at page 6 The Office Action asserts that Risi "teaches prevention of infection in the immunocompromised patient." *Id.* The Office Action concludes that it would have been obvious to one of ordinary skill in the art to combine these references to arrive at the claimed invention. *Id.* at pages 6 and 7.

Applicants respectfully disagree and traverse this rejection.

The claims relate to various methods comprising providing a patient with a composition containing bacteriophage, wherein treatment occurs prior to infection or illness.

As discussed above, the '954 patent does not teach a method comprising treatment of a composition comprising a bacteriophage. Rather, the '954 patent is directed to a method of treating bacterial diseases with lytic ***enzymes***.

Carlton does not remedy the deficiencies of the '954 patent. Carlton generally relates to bacteriophage therapy of patients who have already developed an illness. *See, e.g.*, Carlton, page 271, col. 2.

Risi also fails to remedy the deficiencies of the '954 patent. Among other things, Risi does not teach or suggest the use of bacteriophages.

Applicants submit that one of ordinary skill in the art would have no reason to combine the teachings of the '954 patent, Carlton and Risi. Even if one of ordinary skill in the art had a reason to combine these teachings, which they would not, the combination would not teach each and every limitation. Accordingly, Applicants respectfully submit that a *prima facie* case of obviousness has not been established.

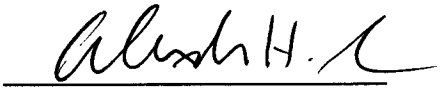
Nonetheless, without acquiescing to the correctness of the rejection, Applicants have amended claims 1, 13 and 32 to recite a bacterial reduction of at least one log. Applicants reserve their right to pursue the subject matter of previous claims 1, 13 and 32 in a continuation application.

CONCLUSION

In view of the foregoing, Applicants respectfully request an indication of allowance of all claims.

Respectfully submitted,

HUNTON & WILLIAMS LLP

Dated: February 15, 2008 By: 

Laurence H. Posorske
Registration No. 34,698

Alexander H. Spiegler
Registration No. 56,625

HUNTON & WILLIAMS LLP
Intellectual Property Department
1900 K Street, N.W., Suite 1200
Washington, DC 20006-1109
(202) 955-1500 (telephone)
(202) 778-2201 (facsimile)

Exhibit A

1. An Overview of the T4 Developmental Program

CHRISTOPHER K. MATHEWS

Analysis of molecular events in T4-infected *Escherichia coli* hosts has revealed some of the most fundamental principles of biology, including (i) relationships between structures of genes and their products, (ii) the existence of mRNA, (iii) virus-induced acquisition of metabolic function, and (iv) morphogenesis of complex structures through sequential gene product interaction rather than sequential gene activation. The T4 life cycle continues to instruct us about biological mechanisms, including those involved in molecular recognition, cooperative functioning of multiple proteins in DNA replication and recombination, processing of mRNA from split genes, mutagenesis, and DNA compaction and packaging.

At one level, an understanding of the T4 life cycle can be seen as a set of answers to questions resulting from a few key experiments, including the following. (i) The one-step growth experiment (Ellis, E. L., and Delbrück, 1939; Doermann, 1948) revealed that the virus is in a noninfectious state for much of its intracellular lifetime (Fig. 1). What is that state? (ii) Hershey (1946b) provided evidence that cells mixedly infected with genetically marked phages yield progeny containing both genotypes, and ultimately recombinant genotypes were identified as well. How do two viral genomes interact within the same infected cell? (iii) Cohen showed that RNA accumulation ceases and DNA accumulation rates increase some 10-fold after infection (see Cohen, S. S., 1968). What accounts for this great increase in the rate of DNA synthesis, and how does RNA transmit information if it is (apparently) not being synthesized? (iv) Induced enzyme synthesis and RNA synthesis in the host were known to be arrested upon infection, and Wyatt and Cohen (1953) showed that DNA synthesized after infection was phage-specific, as revealed by the presence of 5-hydroxymethylcytosine. What mechanisms shut off host cell gene expression, and what pathways account for the synthesis of hydroxymethyldeoxycytidylate (Hm-dCMP) DNA residues? (v) Hershey et al. (1954) showed that T4 proteins labeled early in infection were not incorporated into virus particles, while late-synthesized proteins did label virions. What are the functions of the early proteins? (vi) Infection with replication-defective T4 mutants showed failure of early genetic functions to be shut off late in infection, and failure of late genes to become activated. What factors control the pattern of sequential expression of sets of viral genes?

Before addressing these questions, let us say a bit more about the one-step growth experiment, the structure of the T4 virion, and some features that have made this virus popular with a worldwide scientific community.

As shown in Fig. 1, the one-step growth curve allows partial analysis of a single infective cycle. Aliquots of infected cells are plated on a lawn of susceptible bacteria. Either one phage particle or one infected cell gives rise to one plaque (an "infective center"). Typically, lysis is seen to occur at about 25 min, when the infective-center titer sharply rises, as each infected cell releases 100 to 500 progeny phage. One can also follow the intracellular development of phage by treating each sample with chloroform before plating. This treatment lyses infected bacteria and releases the intracellular contents. Under these conditions each completed phage particle gives rise to one plaque. The most interesting feature of this part of the one-step growth experiment is the eclipse period—approximately the first 12 min, during which time no infective virus can be recovered from the culture, even though each cell is productively infected.

While the structure of the virion is more complex than those of most other bacteriophages, T4 is not qualitatively different from other viruses. Like most viruses, T4 has a genome consisting of a single molecule of nucleic acid. The approximately 172-kbp linear duplex DNA of T4 is one of the largest known bacteriophage chromosomes. Like those of all other viruses, that genome is encapsidated within a protective head, from which the nucleic acid is ejected early in infection. Like many other phages, T4 has attached to its head a tail, which serves as an adsorption organ and a channel for transfer of DNA into the infected cell (see chapter 34). Like several other phages, T4 DNA contains a modified base, which serves in part as a protective device (chapter 36).

What features of T4 biology are distinctive or unique? To begin with, T4 and its close relatives present several technical advantages. First, under suitable infection conditions, plating efficiencies approach 1.0, meaning that virtually every phage particle plated on a lawn of susceptible bacteria can form a plaque. Second, adsorption to host bacteria is rapid, so one can sample cultures that have undergone synchronous infection. Third, T4 uses several mechanisms to arrest the synthesis of nucleic acids and proteins of the host cell. Thus, one can administer isotopic precursors to infected cultures, confident that most or all of the label is flowing into virus-specific macromolecules. Fourth, burst sizes or phage yields are relatively large (usually 200 phage particles or more per cell, produced within 15 to 30 min at 37°C, so infected cells make reasonably large amounts of phage gene products, useful to the experimenter who wants to isolate any of them. The popularity of this phage derives also in large part from the fact that Edgar and Epstein chose T4 for the first large-scale isolation of conditional lethal mutants (Ep-

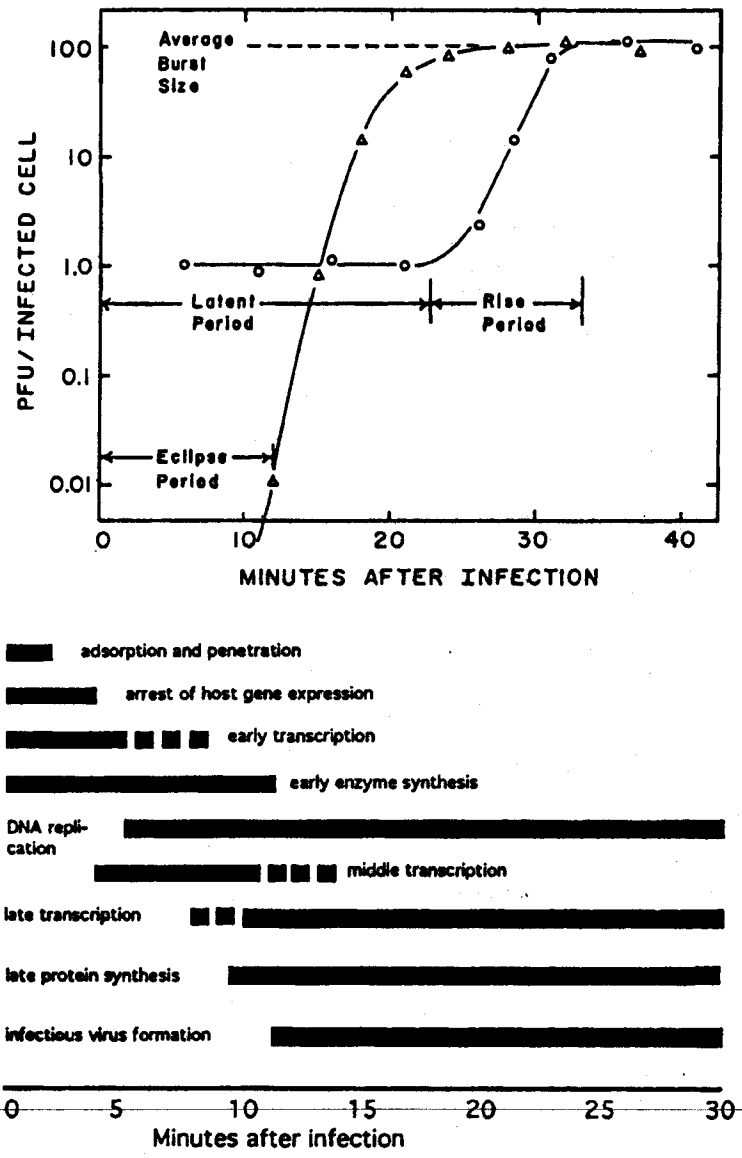


Figure 1. Top, a one-step growth curve for bacteriophage T4D infecting *E. coli* B at 37°C. Circles, infective centers (aliquots of the infected cell culture plated directly on plates seeded with *E. coli* B). Triangles, intracellular phage (obtained by treating each aliquot of culture with chloroform before plating). Bottom, a chronology of major events in the T4 infective cycle. The precise times within which each of the major classes of transcripts are formed vary for individual genes within each temporal class.

stein, R. H., et al., 1963). Thus, from an early date, T4 sported a large collection of mutants defective in most functions essential for virus multiplication.

T4 biology is distinctive in several ways, not all of them technically advantageous—in fact, sometimes quite the contrary! First, the genome is circularly permuted with respect to nucleotide sequence, and about 3 kbp of DNA is repeated at both ends of the DNA molecule; this makes the genome about 169 kbp in length. Second, the modified DNA nucleotides are further modified by the presence of glucose, covalently linked to Hm-dCMP hydroxymethyl groups; this protects the DNA against a host restriction system (Fig. 2). Third, the numbers of viral substructures and different structural proteins (nearly 50) exceed those found even in most other large-tailed phages. Fourth, the T4 genome includes three intron-containing genes (the first reported in any prokaryote; see chapters 7 and 16) and one known gene whose information flow is interrupted at the translational level (chapter 17). Fifth, the virus uses RNA polymerase of the host cell for transcription of all of its genes, but it modifies the host polymerase in many ways, most of which can be related to temporal changes in transcription patterns (chapter 10). Sixth, the virus uses two fundamentally different strategies for initiation of DNA replication,

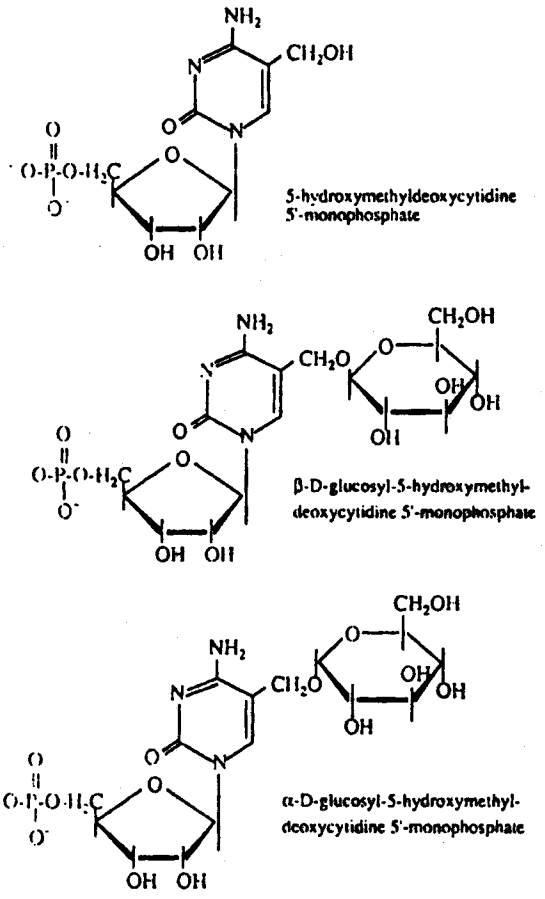


Figure 2. Structures of glucosylated and nonglucosylated Hm-dCMP residues in T4 DNA. Seventy percent of the T4 Hm-dCMP residues are glucosylated in the α configuration, and 30% are glucosylated in the β. T2 DNA is 25% nonglucosylated, 70% α-glucosylated, and 5% diglucosylated (that disaccharide is gentiobiose, β-1,6-glucosyl-α-glucose). T6 DNA is 25% nonglucosylated, 3% α-monoglucosylated, and 72% diglucosylated (also with gentiobiose). See also chapter 36.

one dependent and one independent of discrete replication origins (chapter 4). Seventh, a number of T4-encoded proteins play unexpected dual or multiple roles. Examples include gp63 (product of gene *63*), which serves both as an RNA ligase (of unknown function) and a catalyst for joining tail fibers to baseplates; gppseT, which is both a polynucleotide 5′ kinase and a DNA 3′ phosphatase (both activities of unknown function); and gpalc/unf, which is an RNA polymerase-associated protein that blocks transcription of cytosine-containing DNA, thereby contributing toward the unfolding of the host cell chromosome.

STRUCTURE OF THE VIRION AND EARLY STEPS IN INFECTION

Figure 3 shows the morphology of the T4 particle, with an indication of the number of proteins in each substructure. Below a head possessing quasi-icosahedral symmetry lies a "collar" structure with attached whiskers, not visible in the figure. Normally the long tail fibers extending from the baseplate are extended against the collar, but in the presence of a bacterial cell the fibers are released, which allows the distal tips of the fibers to bind specifically to receptors in the host cell outer membrane: diglucosyl residues in the lipopolysaccharide of *E. coli* B, or the OmpC protein in *E. coli* K-12. The interaction with T4 particles involves the C-terminal sequence of gp37, the protein that forms the distal tip of the long fibers (chapter 22). This is followed by repositioning of the phage so that the baseplate sits above a site of adhesion between inner and outer membranes (Bayer, 1968a). At this point, short tail fibers extending from the baseplate (gp12) "pin" the phage to the cell, via binding to outer membrane lipopolysaccharide. This in turn triggers a still incompletely understood conformational change, the hexagon-to-star transformation in the baseplate. This simultaneously opens a hole in the baseplate large enough for duplex DNA to pass through and triggers rearrangement of gp18 subunits, the tail sheath protein. In turn the tail sheath contracts, forcing the core (composed of gp19) through the host cell surface (see left-hand portion of Fig. 4). One baseplate protein, gp5 (and possibly also gp25), is an active lysozyme, and these activities may facilitate tail penetration by weakening the outer membrane. At any rate, this process is followed quickly by DNA ejection from the head into the interior of the cell. While the mechanism of ejection remains obscure, the process evidently requires (i) maintenance of an electrochemical proton gradient across the membrane, (ii) the establishment of a voltage-dependent ion-selective channel, and (iii) protection of ends of the injected DNA from degradation, by binding gp2, a putative pilot protein.

EARLY METABOLIC EVENTS

Figure 4 summarizes events in the T4 life cycle. Immediately after infection, the unmodified *E. coli* RNA polymerase transcribes a set of genes from promoters that do not resemble those of *E. coli*, but that do compete with host promoters for binding RNA polymerase. The products of these genes include gpalc, an RNA polymerase-binding protein that diverts RNA polymerase from transcribing the cytosine-containing host cell DNA. Other proteins alter the translation apparatus; one of these cleaves a leucine tRNA that translates codons that are abundant in *E. coli* genes but rare in T4 genes. Several T4 tRNAs are synthesized, with codon specificities corresponding to those abundant in T4 late genes. A number of early phage proteins associate with membranes and with ribosomes of the host cell, but the functions of most of these proteins are still obscure, as is a modification of valyl-tRNA synthetase that occurs early in infection.

A number of early T4 proteins act together to completely disrupt and degrade the host cell chromosome. The *ndd* gene product morphologically disrupts the host nucleoid, and inhibition of host cell transcription by the *alc* product leads to unfolding of the independently folded domains of the nucleoid. Several phage-coded nucleases cooperate to degrade *E. coli* DNA to mononucleotides, using the presence of cytosine to dis-

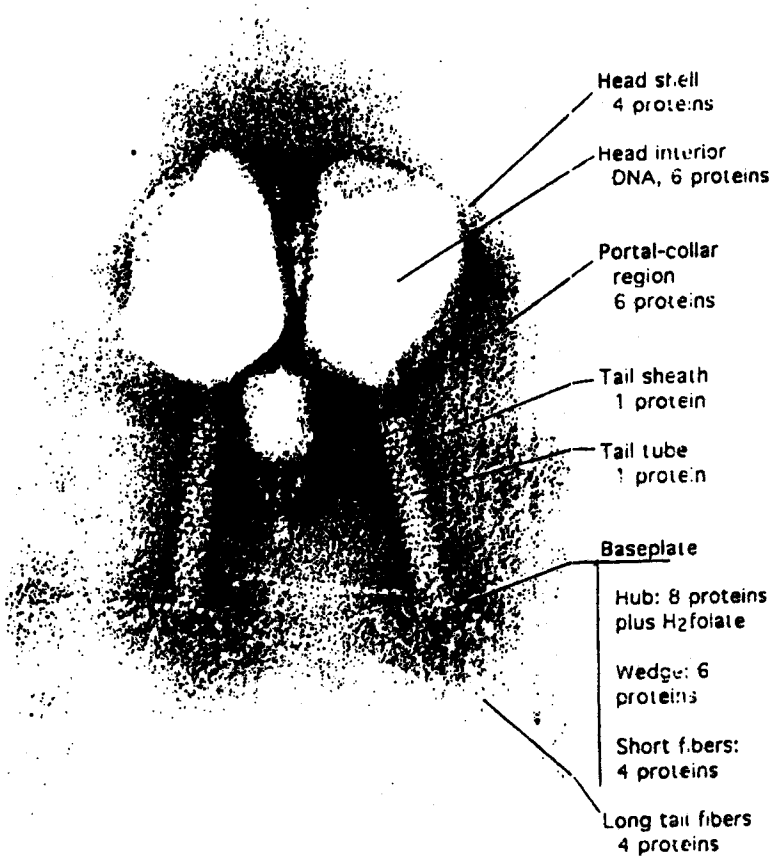


Figure 3. Structure of T4 particles. This "family portrait," taken by Dwight Andersen, shows two T4 particles and one particle of phage ϕ29. Magnification, ca. ×500,000.

tinguish host from phage DNA, which is not degraded. Potentially the host cell contribution to phage DNA nucleotides is about 20 phage-equivalent units per *E. coli* chromosome. About half of the nucleotides released are actually incorporated into DNA of the progeny phage. In addition, ribonucleotides released by host mRNA degradation are efficiently reduced to deoxyribonucleotides and incorporated into DNA.

However, the great bulk of the deoxyribonucleotide building blocks for T4 DNA synthesis comes from de novo synthesis, catalyzed by phage-coded enzymes, which are synthesized within the first 5 min of infection. The dNTP biosynthetic enzymes interact to form a specific multienzyme complex, which may help to shuttle DNA precursors to replication sites (chapter 3). One set of these enzymes, including dCMP hydoxymethylase (gp42), has no host cell counterpart; enzymes in this set catalyze reactions involved in the biosynthesis of the unique hydroxymethyl cytosine nucleotides. The other set, including ribonucleotide reductase and thymidylate synthase, duplicates host cell activities, but augments them greatly and helps support the 10-fold-increased rate of DNA synthesis that occurs after T4 infection.

DNA REPLICATION

The other major class of phage proteins synthesized within the first few minutes consists of those proteins that replicate the phage's DNA: DNA polymerase (gp43), processivity-enhancing proteins (gp45 and gp44 62), single-strand DNA-binding protein (gp32), topoisomerase (gp39, gp52, and gpoo), DNA ligase (gp30), RNase H, and several helicases. DNA replication commences at about 6 min postinfection, with the rate increasing for several minutes. The interactions of proteins at replication forks are quite well understood, because of the development in the laboratories of Alberts and Nossal of excellent in vitro chain elongation systems using purified T4 proteins (chapter 5).

While mechanisms involved in moving the replication fork are similar to those that replicate the host chromosome, T4 is distinctive in that it uses two separate mechanisms to initiate rounds of replication, origin dependent and recombination dependent. The latter processes are subdivided further, into reactions that do and do not involve T4 endonuclease VII (gp49), an enzyme that resolves recombinational intermediates, such as Holliday structures. Biochemical elucidation of these processes has resisted the definition achieved for chain elongation, because of the lack of suitable in vitro systems. Nevertheless, we do know that origin-dependent initiation occurs at several specific sites, each of which involves the action of host RNA polymerase at an early or middle promoter and presumably to synthesize origin-specific RNA primers (chapter 4). A major unanswered question concerns the role of T4 topoisomerase in origin-dependent replica-

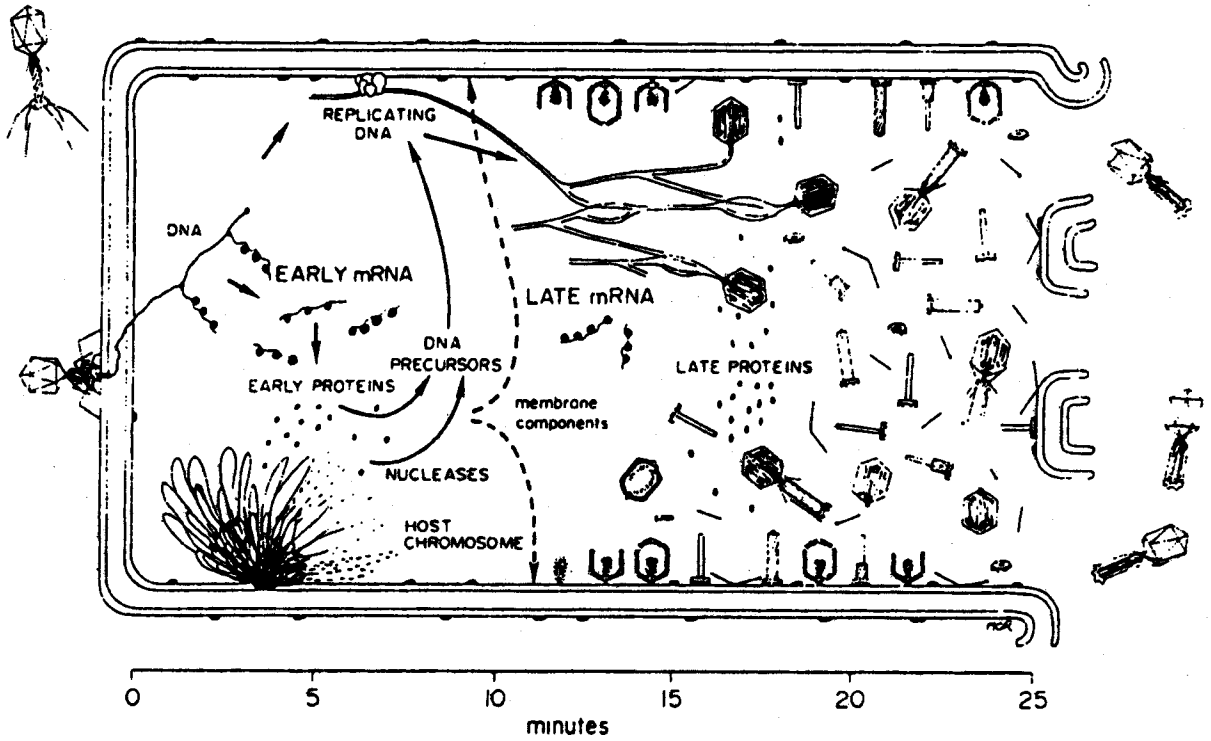


Figure 4. Overview of the T4 developmental program. (Courtesy of Frederick A. Eiserling.)

tion. Early suggestions that DNA underwinding might help to expose single-stranded templates for origin transcription are difficult to reconcile with the lack of DNA gyrase activity for this protein in vitro.

Recombination-dependent initiation requires several gene functions that are also involved in DNA repair and recombination. Mosig has made the reasonable proposal that unreplicated 3′ ends of linear DNA undergoing origin-dependent initiation can invade duplex regions of adjacent genomes and thereby become primers for their own elongation; consistent with this model, recombination-dependent initiation increases late in infection and does not require fixed origin sequences (chapter 4). Although recombination-dependent initiation occurs early in infection, it is distinct from origin-dependent initiation in being rifampin resistant, implying that host cell RNA polymerase is not required.

A redundancy of initiation mechanisms may be necessary to ensure that replication begins under a range of physiological conditions. However, multiple initiation processes may also be essential to increase the rate of DNA synthesis. Werner (1968) calculated that T4-infected bacteria contain as many as 60 replication forks, some 10-fold more than those in a rapidly growing *E. coli* cell. Since replicative DNA chain growth rates are similar in infected and uninfected cells, the repeated initiation of new rounds of replication may be the single most important factor in augmenting the total rate of DNA accumulation per cell.

Because T4 DNA is linear, the T4 genome faces the problem of replicating 3′ ends of parental molecules. It solves the problem by creating concatemers—highly branched multimers with arms up to 20 phage-equivalent units long, created by multiple recombination between chromosome ends and homologous sequences in partner molecules. The nuclease controlled by genes *46* and *47* is involved in this process; mutants that are defective in either gene initiate replication normally but cannot sustain it, because of their inability to recombine and form concatemers. Because recombination systems are highly active in T4 infection, in many cells the entire pool of replicating DNA molecules, up to 100 phage-equivalent units in toto, may consist of one huge DNA molecule.

CONTROL OF GENE EXPRESSION

The fact that virus-specific proteins are synthesized from the earliest time in infection, while virion structural proteins are produced relatively late, implied quite early the existence of temporal patterns of gene expression. The involvement of transcriptional regulation was apparent by the mid-1960s, primarily as the result of DNA-RNA competition hybridization experiments. However, the technology was a bit cumbersome, and most early insights came from analyses of patterns of protein synthesis. In one of the first uses of sodium dodecyl sulfate-polyacrylamide gel electrophoresis coupled with radioautography, Hosoda and Levinthal (1968) showed dramatic changes in protein synthesis patterns at different times after T4 infection. Because the large catalog of existing chain termination mutants allowed identification of many of the proteins seen on one-dimensional gels, much information could be extracted from experiments of the type summarized in

Fig. 5. Later, much additional information could be obtained from the use of two-dimensional gels.

Much more information emerged in succeeding years, with the development of in vitro transcription-translation systems, blotting technology, and subunit analyses of purified RNA polymerase. What emerged was a pattern of regulatory events that is much too complicated to describe appropriately in a few sentences. Transcription of many genes is regulated by multiple mechanisms, and translational mechanisms overlie transcriptional control. For example, several genes whose protein products are produced only late in infection are transcribed early, but their early mRNAs are not translated. Also, several early-expressed genes are transcribed from different promoters at different times in infection.

Mosig and Hall have admirably addressed the biological rationale for much of this regulatory complexity (chapter 10). Nevertheless, a few general statements can be made here. It is now clear that T4 genes are temporally regulated mostly at the level of transcriptional initiation. However, there are important translational control mechanisms as well, notably (i) the translational autoregulation of genes *32* and *43* and (ii) the action of the RegA protein, which binds the translation initiation regions of several mRNAs for early proteins and shuts off translation of these mRNAs. In addition, the T4 tRNA anticodons are those that bind preferentially to codons that are abundant in late T4 messages.

The story of transcriptional regulation is largely a story of changes in phage-coded proteins that associate with *E. coli* RNA polymerase, thereby directing it to transcribe different classes of promoters. However, the story is a bit more complicated, partly because the structure of the DNA template as well as promoter specificities changes with time, partly because many genes are transcribed from different promoters at different times in infection, partly because antitermination mechanisms probably account for some transitions in transcription patterns, and partly because not all structural changes in RNA polymerase have been characterized with respect to effects on transcriptional specificity. What follows is a highly simplified summary of transcriptional regulation (see also Fig. 6).

We recognize three temporal classes of T4 transcripts: early, middle, and late. The simplest basis for classification is that early gene transcription occurs even when protein synthesis is blocked, middle transcription requires some prior phage protein synthesis, and late transcription requires phage protein synthesis and concurrent DNA synthesis. Early transcripts are those that can be synthesized by unmodified RNA polymerase, although, as noted above, promoters for these genes do not closely resemble *E. coli* promoters. However, RNA polymerase does become modified within the first minute or two after infection, and these changes may help to direct RNA polymerase to T4 early promoters. First, one of the two α subunits undergoes ADP-ribosyl transfer at an arginine residue (from NAD^+), through the action of gpalt, a protein that is injected into the host cell along with phage DNA. Later a newly synthesized protein, gpmod, ADP-ribosylates both α subunits. These modifications reduce affinity of the core polymerase for the major host cell σ factor (σ^{70}), and they increase the efficiency of rho factor-dependent termination. The action of another phage gene product,

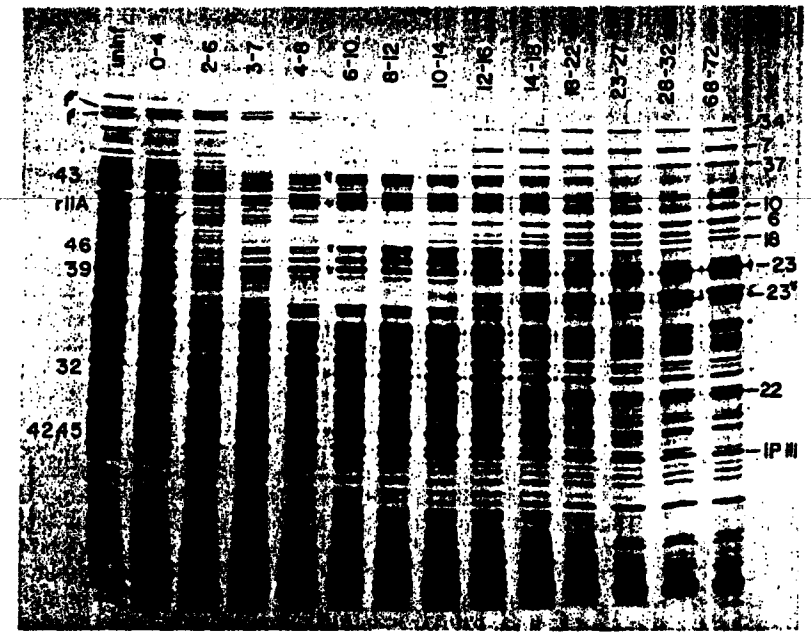


Figure 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and radioautographic analysis of proteins synthesized at different times in the T4 life cycle. Proteins were radiolabeled for 4-min intervals at the indicated times after infection at 30°C. Proteins were resolved on a gradient gel (7.5%–10%–12% steps) and visualized by radioautography. Several major early proteins are marked with asterisks and identified at the left. Late proteins are identified at the right. β and β' are subunits of *E. coli* RNA polymerase. (Courtesy of Jim Karam.)

Polymerase structure	Promoter	Other requirements	Transcript class
$\alpha_2\beta\beta'\sigma^{70}$	GTTTACn14GTGGTAnnAT		Early
↓ gp*alt*, gp*mod*			
↓ RpbA, RpbB			
↓ AsiA			
$(\alpha_{ADPR})_2\beta\beta'$(RpbA,B)	A^T/ATGCTTn13TATAAT	gp*motA* anti-termination factors	Middle
↓ gp33, gp55			
↓ gp45, gp44/62			
$(\alpha_{ADPR})_2\beta\beta'$(RpbA,B) gp33,gp55	TATAAATA (-10)	gp45 gp44/62	Late

Figure 6. Changes in RNA polymerase, promoter specificities, and transcript classes during T4 development.

AsiA (an anti-sigma factor), helps to displace the host cell σ^{70} from RNA polymerase (Orsini et al., 1993). While these modifications may not be essential for T4 growth, they do reshape the enzyme toward its task of transcribing phage genes.

Middle-mode transcription is blocked by inhibition of protein synthesis. Middle transcripts originate either by readthrough from early transcripts (presumably requiring phage-coded antiterminator proteins) or by initiation from middle promoters. Transcription from these middle promoters requires a protein encoded by the *motA* gene. This is a DNA-binding protein that does not bind to RNA polymerase. Instead, it evidently associates with a DNA sequence, TAT/AGCTT, located about 13 nucleotides upstream from the −10 box, and converts this region into an active promoter in the absence of a −35 box.

Late transcription, responsible for synthesis of most virion proteins and assembly factors, requires the most significant changes, both in the structure of RNA polymerase and of the DNA template. RNA polymerase, which by now has lost *E. coli* σ^{70}, becomes associated with two T4 proteins: gp55, a phage-coded σ factor, and gp33, which supports the gp55 function. The consensus late promoter sequence contains no −35 region, only the sequence TATAAATA, located a few nucleotides upstream from the transcription start point. It has long been known that late gene expression in vivo requires concurrent DNA replication, suggesting that only replicating DNA can serve as a transcription template for these genes. Recent developments in Geiduschek's laboratory have shown that proteins of the T4 replication complex serve as a "mobile enhancer" (Herendeen, Kassavetis, et al., 1992). gp45 and the gp44/62 complex have long been known to play essential roles in DNA replication by increasing processivity of the gp43 DNA polymerase. These proteins also track along DNA, interacting directly with template-bound RNA polymerase and stimulating its action at late promoters. It is not yet clear whether the replication proteins encounter RNA polymerase already bound at a late promoter or whether the entire complex of RNA polymerase and DNA polymerase-accessory proteins tracks along until it reaches a late promoter. In any event, these protein-protein interactions are essential, although under some conditions late gene transcription can occur without simultaneous DNA replication.

MORPHOGENESIS AND DNA PACKAGING

Early electron microscopic analysis of T4-infected bacteria revealed that phage substructures—heads, tails, tail fibers—accumulate more or less simultaneously. Moreover, analysis of incomplete structures in the earliest morphogenesis-defective mutants analyzed by R. H. Epstein et al. (1963) showed that when synthesis of one structure—for example, tail tubes—was defective, the other substructures—heads, baseplates, tail fibers—accumulated. These observations suggested that assembly involved not a sequential pattern of gene expression, but rather simultaneous gene expression, with independent pathways of sequential gene product interactions. Direct evidence for this came from the development of in vitro complementation systems by Edgar and Wood (1966). Mixing together of mutant-infected cell extracts, each defective in one assembly function, would lead to formation of infectious virus in vitro if and only if the gene product interaction that resulted from the mixing was an earlier reaction in the assembly pathway than that leading to either partially assembled structure present in the extracts. While there were a few exceptions to this general picture of obligatory sequences of gene product interactions (e.g., gp11 can add late to the developing baseplate), the general picture was as summarized in chapter 18. Note that there are separate and independent subassembly pathways leading to heads, tails, and tail fibers. Assembly of the tail is further divided into separate pathways of baseplate core and wedge substructures, followed in turn by assembly into baseplates, polymerization of tail tubes, and polymerization of tail sheaths.

Other morphogenetic principles emerged from subsequent analysis of the in vitro complementation system: the existence of a few morphogenetic proteins

whose roles are catalytic (e.g., gp38 involvement in fiber assembly and gp63 involvement in fiber-baseplate attachment); the involvement of selective proteolysis of head precursor proteins; the role of some proteins in limiting self-assembly reactions, as inferred from the existence of mutants making polyheads or polysheaths; the involvement in head morphogenesis of scaffolding proteins, such as gp22, that are not found in the mature head; probable interaction between a head morphogenetic protein (gp31) and a chaperone protein of the host cell (GroEL); and the expansion of a head precursor linked somehow to its packaging with DNA.

The latter process, DNA packaging, can be considered both as the last step in DNA metabolism and as an integral step in morphogenesis. The process involves interaction of concatemeric DNA from the replicating, recombining pool with a partially completed head precursor, the prohead. One vertex of the prohead contains a "portal" structure, through which DNA passes both during packaging and later ejection, and to which the tail eventually becomes attached. Concomitant with an expansion of the prohead, some of the DNA enters the structure through the portal. Two enzymatic proteins, gp16 and gp17, are involved. The mechanism is not known, but it involves ATP hydrolysis. Evidence favors an ATP-driven DNA translocation into the prohead. Gp17 binds and requires ATP for function, but identification of the ATPase has not yet been reported.

Whatever the mechanism of DNA packaging, the length of DNA that is packaged is determined by the length of the head. Morphogenetic variants containing abnormally long or short heads exist, and in each case the altered length of the DNA is proportional to the respective increase or decrease in head length. In normal phage morphogenesis the packaged DNA represents about 102% of a genome-equivalent length (172/168.8 × 100%). Cutting a DNA length slightly longer than genome size from a concatemer generates a population of packaged DNA molecules all of which have terminal redundancy and variable endpoints, or circularly permuted base sequences. The terminal repetition ensures that concatemers can form in subsequent infections, even under conditions of single infection, by homologous recombination at the ends, even if the two recombining partners are incompletely replicated at those ends.

LYSIS

The final step in the developmental program is lysis, with release of several hundred newly produced phage particles. Normally, at about 25 min after infection at 37°C, the infected cells die metabolically (i.e., cease production of ATP). This leads to membrane disruption allowing a phage-coded lysozyme to pass through the inner membrane and attack carbohydrates in the outer membrane lipopolysaccharide. This process, which seems conceptually simple, has resisted further elucidation for many years. At least three phage gene products participate, only one of which has a known biochemical function. That protein is the lysozyme encoded by gene *e*, the enzyme that actually degrades the outer membrane. Functions of the other proteins gps and gpt, have not yet been defined biochemically. *t* mutants fail to lyse their hosts, but an *e* mutation can be bypassed under certain conditions if the phage are also mutated in gene *s*. Gpt plays an indispensable role in lysis, probably in disruption of the membrane. *t* mutants fail to lyse their hosts, even if an active *e* gene lysozyme is present.

Several other factors embellish the mysteries surrounding the mechanism and control of lysis. Most important, perhaps, is the phenomenon of lysis inhibition when a previously infected cell is superinfected, the metabolic death of the cell, which leads to lysis, is postponed for up to several hours, and the ultimate phage yields are correspondingly increased. In principle, an understanding of *r*, or rapid-lysis, mutations could help us to understand lysis inhibition; *rI*, *rII*, and *rIII* mutants all display no lysis inhibition. However, for none of these genes has the biochemical function of the gene product been identified. This is especially surprising for the *rII* function, whose genetics occupies a hallowed place in the history of science. Starting with the classic work of Benzer (1959), *rII* mutants have been used for definitive analyses of gene structure, complementation, recombination, mutagenesis, transcriptional regulation, translational control, and membrane biochemistry. Despite all of this attention, we still do not know the biochemical functions of the products of the two *rII* cistrons, nor do we know why the growth of *rII* mutants is restricted in *E. coli* strains lysogenic for phage λ. We do know that the *rII* A and B proteins are membrane bound in infected cells, and they are also found among the proteins retained by a DNA-cellulose column; however, we know little beyond these elementary clues. It is perhaps fitting to end this introductory chapter by highlighting an unsolved problem that is still timely and important, nearly half a century after intensive studies of T4 began. We hope that as you read this book, you will be equally excited with all that T4 has been able to tell us and all that remains to be discovered.